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A possible link between resveratrol and TGF-β: Resveratrol induction of TGF-β expression and signaling

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Abstract **Resveratrol, a polyphenolic compound found in the skin of red fruits, exhibits anti-inflammatory, anti-oxidative, and anti-proliferative characteristics. Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that also displays such properties. We therefore hypothesized that there might be a functional link between resveratrol and TGF-β. This study reports that resveratrol increased transcription of the TGF-β2 gene, enhanced the production of TGF-β2 protein, and activated Smad signaling in an autocrine manner in A549 human lung epithelial cell line. Thus, some of the beneficial effects of resveratrol on human health might be mediated, in part, through its effects on TGF-β expression and signaling.**

Keywords: Resveratrol; TGF-β; Smad; Lung epithelial cells

1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a polyphenolic compound that is abundant in grapes and red wine [1,2]. Resveratrol has been reported to exhibit anti-inflammatory, anti-oxidative, and anti-carcinogenic (anti-proliferative) properties by multiple molecular mechanisms [1–4], including acting as a free radical scavenger. In addition, the structure of resveratrol is similar to the structure of estradiol, and resveratrol acts as an estrogen agonist or antagonist depending on experimental conditions [5,6].

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that is involved in a variety of cellular activities (proliferation, differentiation, extracelluar matrix regulation, and survival) [7]. TGF-β is secreted as inactive (latent) precursors into the extracellular space and is activated by several mechanisms which are not fully understood [8]. TGF-β signals through two serine/threonine kinase receptors (termed type I and type II) and the Smad family of proteins [9]. The type I receptor is activated by the type II receptor upon ligand binding and induces the phosphorylation of Smad2 and Smad3, which in turn form complexes with Smad4. The resulting Smad complex then moves into the nucleus, binds to the Smad-binding elements located in the promoter region of the target genes, and regulates their transcription.

Through its pleiotropic effects on many types of cells, TGF-β has been shown to exhibit anti-inflammatory, anti-oxidative, and anti-carcinogenic (anti-proliferative) effects [8,10–12]. For example, TGF-β1 null mice die of massive inflammatory lesions in many organs immediately after birth [10] and TGF-β displays a strong anti-mitogenic activity in epithelial and hematopoietic cells [12]. Because these properties of TGF-β overlap with those of resveratrol, we hypothesized that TGF-β might be involved in some of the beneficial effects that have been ascribed to resveratrol. To test the hypothesis, this study investigated whether resveratrol, at concentrations that can be attained by red wine intake (1–10 μM) [1], affects TGF-β expression and signaling in several human cell lines.

2. Materials and methods

2.1. Reagents

Resveratrol and HTS466284, a selective small molecule inhibitor of TGF-β type I receptor kinase [13], were purchased from Calbiochem (San Diego, CA). 4-Hydroxytamoxifen (Tamoxifen), ICI182780 (Fluvestrant), and 17-β estradiol were purchased from Sigma–Aldrich (St. Louis, MS). Recombinant human TGF-β1 and anti-human TGF-β type II receptor antibody were purchased from R&D Inc. (Minneapolis, MN).

2.2. Cell culture

The human alveolar carcinoma cell line A549 [14] (RIKEN Cell Bank, Ibaragi, Japan) was maintained in DMEM medium (Invitrogen/Gibco, Carlsbad, CA) containing 10% FCS and antibiotics.

2.3. Reporter plasmid

$(CAGA)_{12}$-luciferase reporter plasmid, which is exclusively activated by TGF-β-induced complex between Smad3 and Smad4, has been described previously [15]. TGF-β-inducible plasminogen activator inhibitor-1 (PAI-1) promoter-luciferase reporter plasmid has been previously described [15]. Human TGF-β2 promoter-luciferase reporter plasmid was generated by inserting DNA fragments of the promoter regions of the human TGF-β2 gene (−639 to −69 from the transcriptional initiation site) into the pGL3-basic vector (Promega, Madison, WI) as previously described [16].

2.4. Transcriptional reporter assay

Cells were seeded at 3×10^4/well in 24-well plates. We then transfected the cells with 200 ng of each reporter construct and 5 ng of pRL-TK Renilla luciferase vector (Promega, Madison, WI), an internal

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control for transfection efficiency, using FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 12 h, the cells were stimulated with 0.1% DMSO, 3 or 10 μM resveratrol, 10 pM estradiol, or 10 ng/ml TGF-β1 with or without 10 nM 4-hydroxytamoxifen or 1 μM ICI182780. Ninety-six hours after the stimulation, the firefly and Renilla luciferase activities were measured as previously described [16].

2.5. TGF-β2 ELISA

The amounts of TGF-β2 in the culture supernatants were determined using the human TGF-β2 ELISA kit (R&D, Minneapolis, MN) according to the manufacturer's instructions. For determination of the total TGF-β concentrations, the samples were activated by an acidification procedure prior to the ELISA assay.

2.6. Quantitative real-time PCR

Quantitative PCR analysis using cDNAs from the A549 cell line specimens was performed using the AB7500 real-time PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions using primers and probes for human TGF-β2 and β-actin (Applied Biosystems).

2.7. Western blotting

Whole cell extracts (10 μg) were subjected to immunoblotting with anti-phosphorylated Smad3 antibody or Smad2/3 antibody (Cell Signaling Technology Inc., Danvers, MA) as previously described [17].

2.8. Data analysis

The data are summarized as the means ± S.D. The unpaired Student's *t*-test was used for the statistical analysis of the results. $P < 0.05$ was considered to be significant. Representative results of three independent experiments are shown.

3. Results and discussion

3.1. Resveratrol increases TGF-β2 expression in A549 human lung epithelial cells via estrogen receptors

To investigate whether resveratrol affects TGF-β expression, we initially examined its effects on the TGF-β2 promoter-luciferase reporter construct that contains the promoter regions of the human TGF-β2 gene in several human epithelial cell lines including HaCaT (keratinocyte cell line), PANC-1 (pancreatic cancer cell line), HeLa (uterus cancer cell line), ZR75-1 (breast cancer cell line), and A549 (lung epithelial cell line). Among the cell lines tested, both 3 and 10 μM resveratrol caused a significant increase in the reporter activity only in A549 human lung epithelial cell line (Fig. 1A and data not shown). In addition, the ELISA assay revealed that the amounts of total TGF-β2, but not TGF-β1 and -β3, protein increased after stimulation with 3 and 10 μM resveratrol in A549 cells (Fig. 1B and data not shown). We confirmed that both 10 and 30 μM concentrations of resveratrol did not affect the cell viability in A549 cells (data not shown).

Resveratrol is structurally similar to estradiol and acts as an estrogen agonist or antagonist depending on the experimental conditions [5,6]. Although the presence of estrogen receptors in human lung tumors has been controversial for many years, Stabile et al. recently demonstrated that some forms of estrogen receptor α and β are indeed present in normal lung cells as well as in lung cancer cells, including A549 cells [18]. To determine whether the resveratrol-induced TGF-β2 expression in A549 cells was via estrogen receptors, the effects of the estrogen receptor antagonist 4-hydroxytamoxifen on the resveratrol-induced TGF-β2 expression were examined in A549 cells. An ELISA assay showed that 4-hydroxytamoxifen

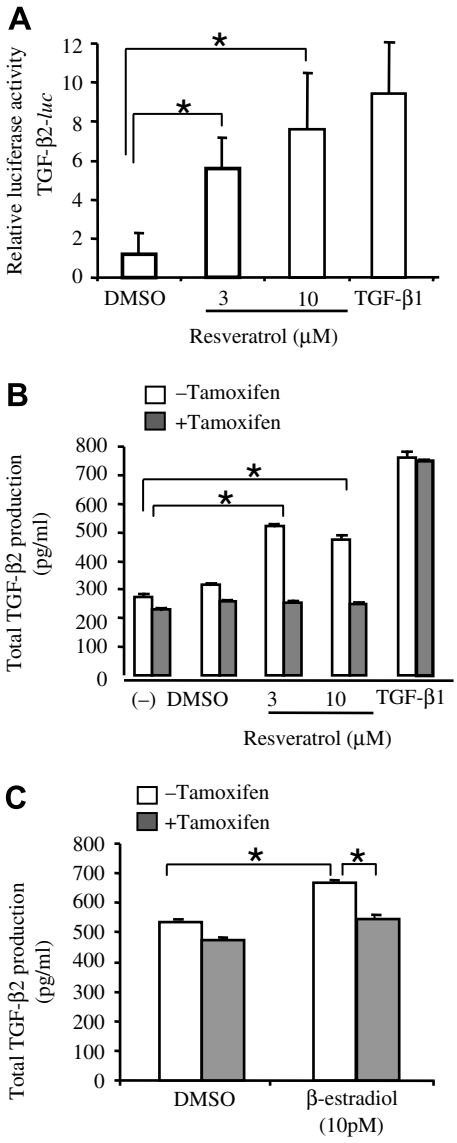

Fig. 1. Resveratrol upregulates TGF-β2 expression in A549 human lung epithelial cells via estrogen receptors. (A) A549 cells were transfected with TGF-β2 promoter-reporter plasmid and stimulated with the indicated doses of resveratrol, control DMSO, or 10 ng/ml TGF-β1. Ninety-six hours after the stimulation, the luciferase activity was measured. The relative activity was calculated by assigning the relative luciferase activity of DMSO a value of 1. (B) and (C) A549 cells were cultured in a medium containing 10% FCS in the presence or absence of the indicated doses of resveratrol, control DMSO, or 10 ng/ml TGF-β1 with or without 10 nM 4-hydroxytamoxifen (Tamoxifen) (B) or in the presence or absence of 10 pM estradiol or control DMSO with or without 10 nM 4-hydroxytamoxifen (Tamoxifen) (C) for 96 h and the concentrations of total TGF-β2 in the supernatants were estimated by ELISA. Each experiment was conducted in triplicate for each sample, and the results are expressed as means ± S.D. $^{*}P < 0.05$.

(Tamoxifen) significantly inhibited the resveratrol-induced increase of TGF-β2 protein in A549 cells (Fig. 1B). We also discovered that 10 pM estradiol stimulated the production of TGF-β2 protein in A549 cells, and this production was blocked with 4-hydroxytamoxifen (Fig. 1C). These results suggest that resveratrol, as an estrogen agonist, induced the formation of TGF-β2 protein through estrogen receptors in A549 cells.

3.2. Resveratrol increases TGF-β/Smad-dependent promoter activity in A549 human lung epithelial cells in an autocrine manner

Because resveratrol increased TGF-β2 expression in A549 cells (Fig. 1), we investigated whether the resveratrol-induced TGF-β2 activates TGF-β signaling in A549 cells in an autocrine manner. The TGF-β-inducible $(CAGA)_{12}$-luciferase reporter construct and the plasminogen activator inhibitor-1 (PAI-1) promoter-luciferase reporter constructs were transfected into A549 cells and then the transfected cells were stimulated with resveratrol. Notably, 10 μM resveratrol significantly increased the luciferase activities of the $(CAGA)_{12}$-luciferase reporter construct and both 3 and 10 μM resveratrol significantly increased the PAI-1 promoter-luciferase reporter construct in A549 cells (Fig. 2A and B). In comparison, TGF-β1 caused an almost 100-fold increase in the $(CAGA)_{12}$ promoter activity in A549 cells (Fig. 2C).

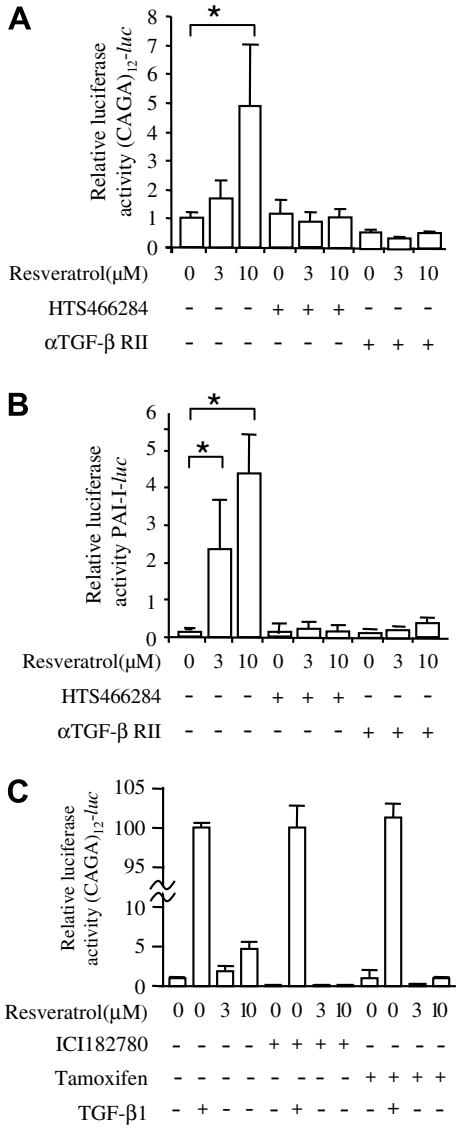

Fig. 2. Resveratrol increases TGF-β/Smad-dependent promoter activity in A549 human lung epithelial cells via endogenous TGF-β. (A) and (B) A549 cells were transfected with $(CAGA)_{12}$-luciferase reporter plasmid (A) or with PAI-1 promoter-luciferase reporter plasmid (B) and stimulated with 3 or 10 μM of resveratrol or control DMSO with or without 10 μM HTS466284 or 10 μg/ml anti-TGF-β type II receptor antibody (αTGF-β RII). Ninety-six hours after the stimulation, the luciferase activity was measured. (C) A549 cells were transfected with $(CAGA)_{12}$-luciferase reporter plasmid and stimulated with 3 or 10 μM of resveratrol or control DMSO with or without 10 nM 4-hydroxytamoxifen (Tamoxifen), 1 μM ICI182780, or 10 ng/ml TGF-β1. Ninety-six hours after the stimulation, the luciferase activity was measured. Relative activity was calculated by assigning a value of 1 to the relative luciferase activity of DMSO control. Each experiment was conducted in triplicate for each sample and the results are expressed as means ± S.D. $^{*}P < 0.05$.

To determine whether the TGF-β2 that is induced by resveratrol contributes to the increased $(CAGA)_{12}$- and PAI-1 promoter-luciferase reporter activities in A549 cells in an autocrine manner, we then examined the effects of the anti-TGF-β type II receptor antibody and HTS466284, a TGF-β type I receptor kinase inhibitor [13], on the resveratrol-induced increase of the $(CAGA)_{12}$- and PAI-1 promoter-luciferase reporter activity. Both the anti-TGF-β type II receptor antibody and HTS466284 significantly reduced the increase in $(CAGA)_{12}$- and PAI-1 promoter-luciferase reporter activity mediated by resveratrol in A549 cells (Fig. 2A and B). In addition, 4-hydroxytamoxifen (Tamoxifen) and ICI182780, another selective inhibitor of estrogen receptor, inhibited resveratrol-induced increase of the $(CAGA)_{12}$-promoter-luciferase reporter activity (Fig. 2C). These results suggested that resveratrol increases TGF-β/Smad-dependent promoter activity in A549 human lung epithelial cells via the induction of endogenous TGF-β and also via the estrogen receptor.

3.3. Kinetics of TGF-β2 mRNA expression and Smad3 phosphorylation following the stimulation with resveratrol in A549 human lung epithelial cell line

The above results suggest that resveratrol initially up-regulates TGF-β2 expression via estrogen receptor and then the endogenously produced TGF-β2 activated Smad signaling in an autocrine manner in A549 cells. To confirm that resveratrol indeed up-regulates TGF-β2 and then activates TGF-β/Smad signaling in A549 cells, the kinetics of TGF-β2 mRNA in A549 cells following the stimulation with resveratrol were examined because TGF-β is a strong inducer of transcription of TGF-β gene itself [19] (Fig. 3A). The TGF-β2 mRNA expression was initially up-regulated at 15 min after the stimulation with resveratrol, following the gradual decrease. Starting at 4 h, the TGF-β2 mRNA expression again increased in A549 cells with its peak at 72 h after the stimulation. In contrast, the stimulation with TGF-β1 rapidly (at 15 min) increased TGF-β2 mRNA expression without any further increase (Fig. 3A and data not shown). These results suggest that resveratrol indeed up-regulates TGF-β2 and then activates TGF-β/Smad signaling in A549 cells, resulting in the second wave of increase in TGF-β2 mRNA levels.

Consistent with the real-time PCR data, resveratrol-induced phosphorylation of Smad3 was observed at 4 h after the stimulation, whereas TGF-β-induced phosphorylation of Smad3 was rapidly observed at 15 min after the stimulation in A549 cells (Fig. 3B). The resveratrol-induced phosphorylation of Smad3 was inhibited by HTS466284, 4-hydroxytamoxifen (Tamoxifen) and ICI182780 in A549 cells (Fig. 3C). These findings suggest that resveratrol-induced phosphorylation of

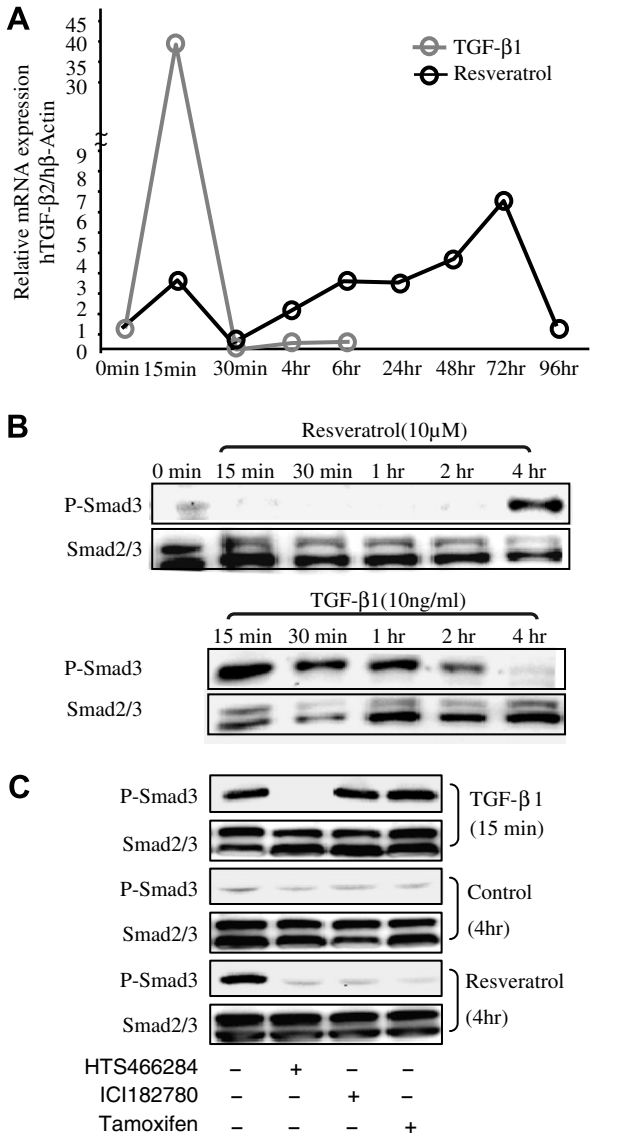

Fig. 3. Kinetic of TGF-β2 mRNA expression and Smad3 phosphorylation following stimulation with resveratrol in A549 human lung epithelial cells. (A) A549 cells cultured in DMEM containing 0.1% FCS were stimulated with 10 μM resveratrol or 10 ng/ml TGF-β1 for the indicated times. RNA was then extracted and real-time PCR for TGF-β2 and β-actin was performed. The ratio of each gene to that of β-actin was calculated, and the value of 1 was assigned to A549 cells at 0 min. (B) and (C) A549 cells cultured in DMEM containing 0.1% FCS were stimulated with 10 μM resveratrol or 10 ng/ml TGF-β1 with or without 10 μM HTS466284, 10 nM 4-hydroxytamoxifen (Tamoxifen), or 1 μM ICI182780 for the indicated times. Western blot analysis with specific antibodies against human phosphorylated Smad3 and Smad2/3 was then performed.

Smad3 in A549 cells was estrogen receptor-dependent and observed at the later time points when endogenous TGF-β2 mRNA was induced, also supporting the earlier results.

Resveratrol-induced TGF-β2 expression and TGF-β/Smad-dependent promoter activation were relatively low in comparison to those induced by TGF-β1 in A549 cells (Figs. 1 and 2). Since TGF-β2 is produced by a latent form, the weak effect of resveratrol may be due to requirement of an activation step for latent TGF-β2, compared to the addition of exogenous ligand. Resveratrol can be consumed daily through wine or fruit, and the weak activity of resveratrol on TGF-β expression and signaling might ensure the safety of resveratrol consumption and prevent the unfavorable effects of TGF-β such as fibrosis. We speculate that long-term intake of resveratrol by wine or fruit may cause a very slight change in TGF-β expression and signaling activity, resulting in beneficial effects on human health.

Lu et al. reported that >5 μM resveratrol-induced TGF-β2 mRNA expression in an estrogen receptor-positive human breast cancer cell line (MCF-7) [20].

Our findings have provided a novel insight into the mechanism underlying their observation and extended their study in that (1) the effects of resveratrol on TGF-β2 expression was via estrogen receptors and was relatively weak in comparison to the effects of other stimulations (TGF-β and estradiol), (2) resveratrol activated TGF-β/Smad signaling through TGF-β in an autocrine manner and estradiol-induced TGF-β2 in A549 cells.

Based on our current results, it can be presumed that the biologically active form of TGF-β2 is generated in A549 cells after stimulation with resveratrol. The mechanisms underlying the conversion of the latent precursors to the biologically active form of TGF-β are complex and are not yet fully understood [8]. Further study is needed to determine how resveratrol induces the active form of TGF-β in A549 cells.

In summary, we showed that endogenous TGF-β, which is induced by resveratrol via estrogen receptors, activated TGF-β/Smad signaling in an autocrine manner in A549 cells. Thus, the effects of resveratrol on TGF-β expression and signaling might contribute, at least in part, to the beneficial effects of this molecule.

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